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Phagocytic Activity of Tunic Cells in the Colonial Ascidian *Aplidium yamazii* (Polyclinidae, Aplousobranchia)

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ABSTRACT—The phagocytic activity of tunic cells of the colonial ascidian *Aplidium yamazii* was assessed by incubation of thin tunic slices including these cells with fluorescent microparticles. Only one type of tunic cell engulfed the microparticles. These phagocytic tunic cells are irregularly shaped, motile, and often contain phagosomes. Many of them also contain vesicles laden with round granules. Occasionally, they engulf another tunic cell. Because this type of tunic cell is always found in the tunic of histological sections prepared from whole (unsliced) colony specimens, these cells are probably distributed in the tunic under normal conditions. Peroxidase activity was demonstrated exclusively within granule-containing vesicles of some phagocytic tunic cells. This finding indicated that the phagocytic tunic cells might possess an oxygen-dependent microbicidal system. It is presumed that the phagocytic tunic cells migrate throughout the tunic matrix, engulf extraneous substances (including bacteria) and also function as scavengers to keep the tunic free of discarded tunic cells and other debris, such as from wounds.

INTRODUCTION

The tunic is an integumentary tissue in urochordates, such as ascidians; it is a gelatinous or leathery matrix covering the outer surface of epidermis. The tunic is a unique tissue in animals because of its cellulosic component [1,12]. The main function of the tunic is protection of the body, although little is known about this defense system. The tunic is an attractive material for studying defense systems from the viewpoint of comparative immunology because of its peculiarity and the phylogenetic position of ascidians, whose ancestors may be the same as those of the vertebrates.

Ascidians have two types of free cells: hemocytes and tunic cells. Hemocytes circulate in the blood vessels and in the mesenchymal space, and tunic cells are distributed in the tunic; both cell types are presumed to have immunological activity. The phagocytic activity of these free cells was mainly studied by injection or insertion of foreign substances (reviewed in [13]). These studies could not determine whether one or both of these cell types was involved in phagocytosis, because hemocytes seem to infiltrate the tunic that is responding to the experimental operations. Recent studies have demonstrated that particular types of hemocytes have phagocytic activity *in vitro* [9, 10, 14]. As for tunic cells, De Leo *et al.* [2] described the "phagocyte" that is characterized by one or two "heterolysosomic vacuoles" as a type of tunic cell in the solitary ascidian *Ciona intestinalis*. Parrinello *et al.* [5, 6, 7] reported on the phagocytic activity and inflammatory reaction in the tunic by introducing foreign substances into tunic. However, dealing with tunic cells has following difficulties: It is almost impossible to isolate the tunic cells from tunic matrix, and the specimens are usually contaminated by leakage or infiltration of hemocytes.

Aplidium yamazii, a colonial ascidian belonging to the family Polyclinidae and the suborder Aplousobranchia, forms a white, sheet-like colony, and its elongated zooids are separately embedded in a transparent, gelatinous tunic. When a live colony is cut into slices of about 0.5 mm thick, the tunic cells in the slices are observable under a light microscope and remain alive for several hours to a few days. Using these live colony slices, the present study experimentally demonstrated phagocytosis of fluorescent microparticles in the tunic. In this study, the possibility of hemocyte contamination was almost eliminated, because this polyclinid species has no blood vessels in the tunic. We also performed cytochemical determinations for peroxidase, which may be involved in microbicidal activity.

MATERIALS AND METHODS

Animals

Colonies of *Aplidium yamazii* were collected in Nabeta Bay, Shimoda (Shizuoka Pref., Japan). They were attached to glass slides with cotton thread and were reared in culture boxes immersed in Nabeta Bay. The sheet-like colonies grew and spread on the glass slides (Fig. 1A).

Assay for phagocytic activity

A growing part of a live colony was cross-sectioned into slices about 0.5 mm thick using a razor blade (Fig. 1B). Each colony slice consisted of the gelatinous, transparent tunic, the tunic cells, and some fragments of zooids. A solution of fluorescent microparticles (fluoresbrite carboxylate microspheres, 0.5 μm in diameter, 2.5%

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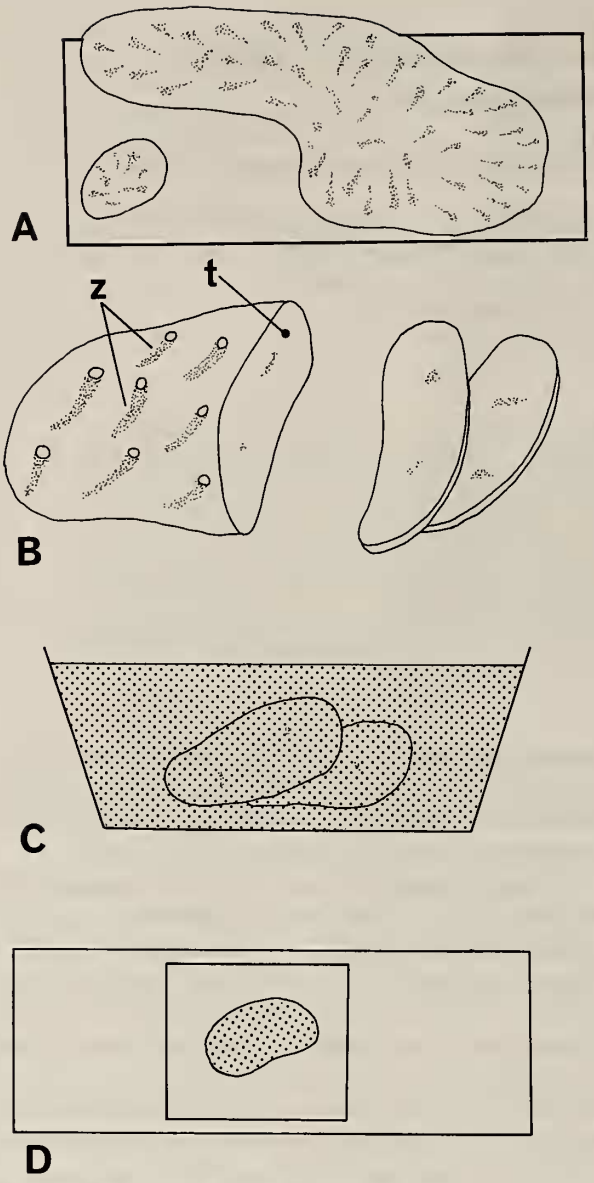


Fig. 1. The procedures of the assay for phagocytic activity. t, tunic; z, zooids. A: Colonies of *A. yamazii* growing on a glass slide. B: Sectioning of a live colony. C: Incubation of colony slices in filtered seawater containing fluorescent microparticles. D: Mounting a colony slice on a glass slide.

solid latex; Polyscience) was diluted in 1:5 with filtered seawater (FSW). The colony slices were incubated in this solution for 30 min at room temperature, allowing the microparticles to permeate the tunic (Fig. 1C). After extensive washing with FSW, the slices were incubated in FSW or FSW containing penicillin (100 IU/ml) and streptomycin (1 mg/ml) for 1–2 hr, 24 hr, or 48 hr at room temperature. Because the specimens that were incubated for 24 hr or 48 hr markedly shrank, they were sliced again for microscopic observation. Each slice was observed under a light microscope equipped with epifluorescence and Nomarski differential interference contrast (DIC) optics (Fig. 1D).

For electron microscopy, some colony slices incubated with microparticles were fixed in 2.5% glutaraldehyde-0.1M sodium cacodylate-0.45 M sucrose (pH 7.4) for 2 hr on ice. They were rinsed in 0.1 M sodium cacodylate-0.45 M sucrose (pH 7.4), and postfixed in 1% osmium tetroxide-0.1 M sodium cacodylate (pH 7.4) for 1–1.5 hr on ice. They were dehydrated through a graded ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low viscosity epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined using a Hitachi HS-9 transmission electron microscope at 75 kV.

We also fixed colony pieces of about 5 mm×10 mm (containing more than 30 zooids) and processed them for electron microscopy as described above. We wanted to determine whether the phagocytic cells observed in the above experiment were always distributed in the tunic or were contaminating hemocytes from the slicing process.

Cytochemistry for peroxidase activity

The colony pieces were fixed in 2.5% glutaraldehyde-2% NaCl-0.1 M Millonig's phosphate buffer (pH 7.4) on ice for 15 min, and the fixed pieces were cut into slices with a razor blade. After washing in the same buffer, they were pre-incubated in 0.1% diaminobenzidine (DAB; Sigma)-0.1 M phosphate buffer (pH 6.8) for 30 min at room temperature and then incubated in 0.1% DAB-0.3% H_2O_2-0.1 M phosphate buffer for 15 min. The specimens were washed with 0.1 M phosphate buffer and postfixed with 1% osmium tetroxide. They were dehydrated and embedded in epoxy resin as described above for electron microscopy. Thick sections were stained with toluidine blue. In negative controls, we omitted H_2O_2 from the incubation medium or added 50 mM 3-amino-1,2,4-triazole (Sigma) in the pre-incubation and incubation media as an inhibitor.

RESULTS

In the tunic of colony slices of *Aplidium yamazii*, there were tunic cells of various types; some types have protruding filopodia, some types have an elongated cell shape, some contain many granules, and some form multicellular vesicles. Particular types of tunic cells showed phagocytic activity (Fig. 2). Although these phagocytic cells were found throughout the tunic matrix, they did not appear to be evenly distributed. This was caused by the uneven distribution of microparticles within the tunic slices; that is, there were more microparticles per unit area at the surface of the slices than at the core region. For this reason, we could not make a quantitative description of the distribution of phagocytic tunic cells in this study. The tunic cells phagocytizing microparticles were essentially irregularly shaped cells with extending filopodia. There was, however, some variation in their appearance. For instance, Figure 3 shows three tunic cells, all of which engulfed the microparticles. Cell "a" was a thin, flattened cell with numerous filopodia; cell "b" was almost round; and cell "c" was elliptical. Cells "b" and "c" had thicker cell bodies and fewer filopodia than cell "a". Because many tunic cells that exhibit phagocytosis showed intermediate appearances among these three, we classified them as a single cell type, namely, phagocytic tunic cells. These cells occasionally engulfed another tunic cell, and thus might be considered scavengers. A tunic cell engulfing a relatively large cell did not have prominent filopodia, and its cell body became a thin sheet that wrapped around the engulfed cell. With respect to the characteristics of phagocytic cells (i.e. cell

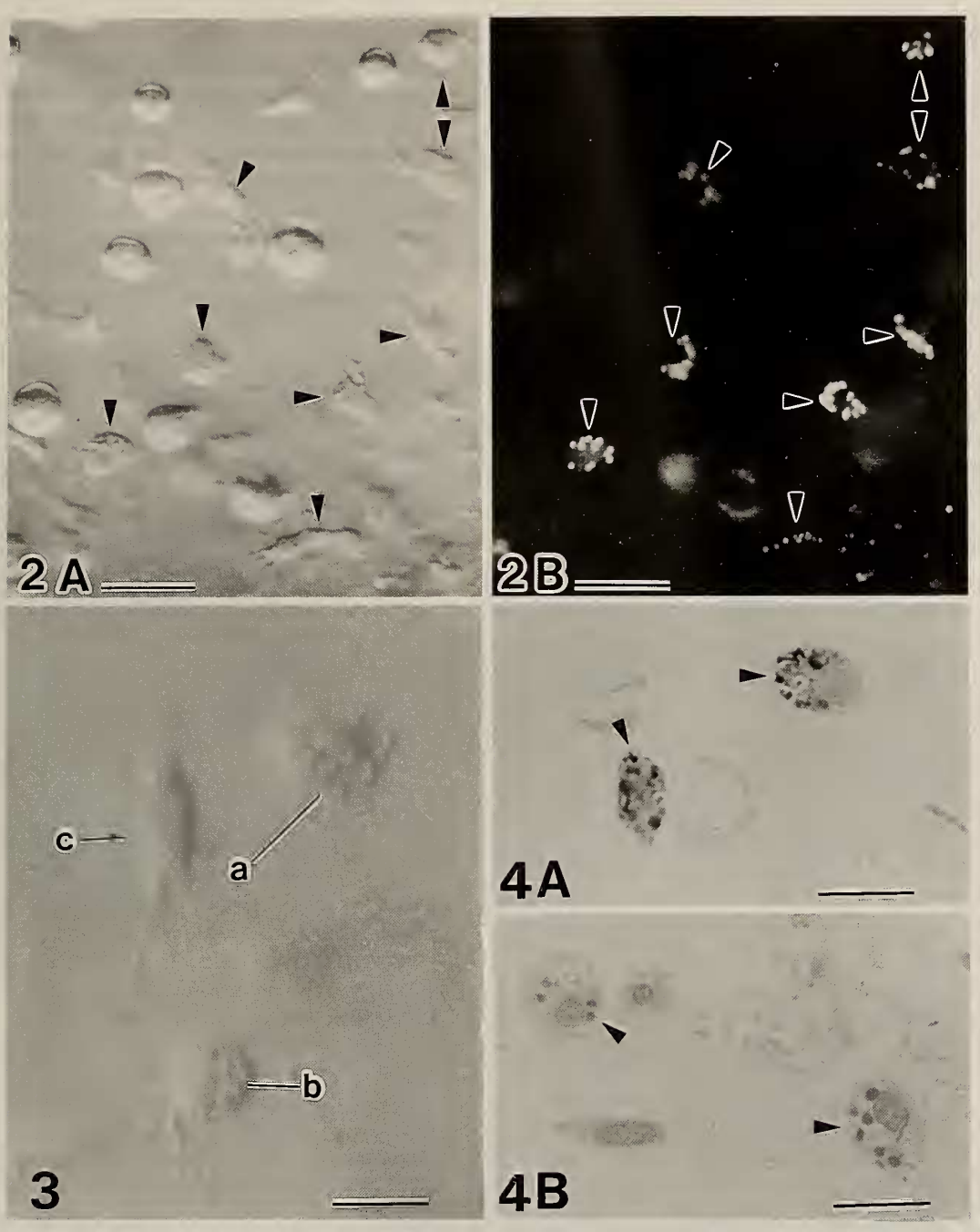


FIG. 2. Paired images of a live colony slice incubated for 2 hr in FSW. A: Nomarski DIC. B: Epifluorescence. Arrowheads indicate tunic cells phagocytizing fluorescent microparticles. Note round granular cells and some thin filopodial cells do not contain the microparticles. (Scale bar=25 μm)

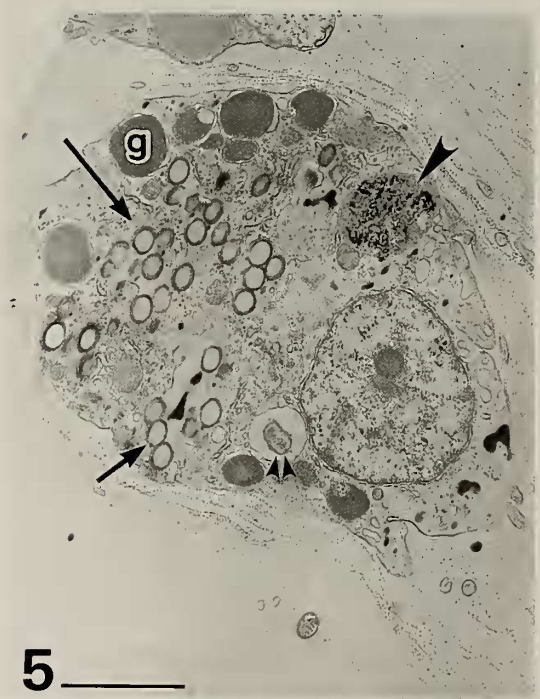
FIG. 3. Light micrograph (Nomarski DIC) of three phagocytic tunic cells (a, b, c) in live colony slice incubated for 2 hr in FSW. Each cell has a different appearance from the others with respect to cell shape, thickness of cell body, and number of filopodia. (Scale bar=10 μm)

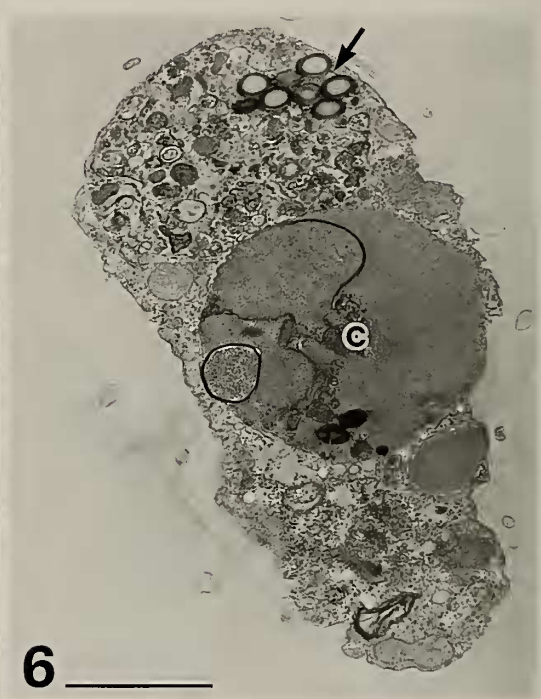
FIG. 4. Cytochemistry for peroxidase in the tunic. A) Dark reaction product indicates peroxidase activity in phagocytic tunic cells (arrowheads). B) A negative control in which peroxidase inhibitor (3-amino-1,2,4-triazole) was added. The reaction product is not found in any tunic cells. The granular inclusions are only stained with toluidine blue (arrowheads). (Scale bar=10 μm)

shape, granular inclusions, and distribution of engulfed microparticles within the cells), prominent differences were not found among the specimens incubated for 2 hr, 24 hr, and 48 hr. In preliminary observations using time-lapse video recording, these phagocytic tunic cells actively migrated within the tunic matrix and some non-phagocytic ones did not. The ability to migrate may be indispensable for phagocytic activities.

In the cytochemical study, only some of the phagocytic tunic cells showed peroxidase activity. In thick sections for light microscopy, the dark product indicating peroxidase activity is uniquely localized within the phagolysosome-like vesicles and/or vesicles carrying round granules (Fig. 4A). No specific activity is demonstrated in the other cell compartments or in the other types of tunic cells. For example, a phagocytic cell engulfing another cell has peroxidase activity in its vesicles that contain round granules, but not in the large phagosome that engulfs the cell. No peroxidase activity was demonstrated in the negative controls (Fig. 4B).

In electron microscopic observations, the microparticles were recognized as electron-lucent rounds or ellipsoids whose margins were moderately electron dense. Some microparticles adhered to exposed surface of the tunic matrix, and some permeated the tunic slices. In phagocytic tunic cells engulfing the microparticles, the latter were found within the vesicles, and these cells also had some vesicles laden with round, electron-dense granules (Figs. 5 and 6). The largest of these granules were about 1.5 μm in diameter, with some





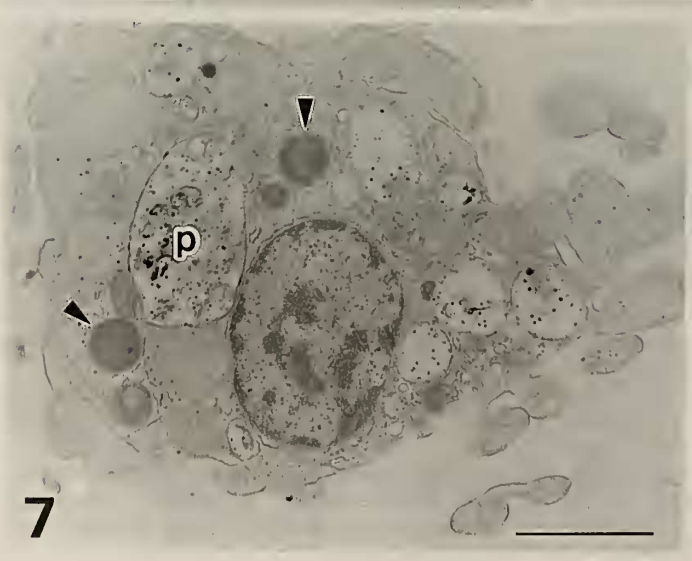


Fig. 5. A phagocytic tunic cell in the colony slice incubated for 2 hr in FSW. The cell contains granules (g), the engulfed microparticles (arrows) and a phagolysosome-like vesicle (arrowhead). This cell also carries a vesicle containing a bacteria-like structure (double arrowheads). (Scale bar=2 μm)

Fig. 6. A phagocytic tunic cell in the colony slice incubated for 24 hr in FSW. The cell engulfs microparticles (arrow) and another tunic cell (c). (Scale bar=2 μm)

Fig. 7. A phagocytic tunic cell in a specimen fixed as a whole colony piece. The cell contains electron-dense granules (arrowheads) and phagolysosome-like vesicles (p). (Scale bar=2 μm)

being discernible under the light microscope. Some of the phagocytic tunic cells also had phagolysosome-like vacuoles containing disorganized structures that appeared to be disintegrating cellular components. In addition, a bacteria-like structure was occasionally found in a vesicle (double arrowheads in Fig. 5). In Figure 6, the phagocytic tunic cell carrying microparticles has engulfed another tunic cell. Tunic cells with the same characteristics described above were also found in specimens fixed as whole colony pieces (Fig. 7).

DISCUSSION

When colony slices of *Aplidium yamazii* are incubated with microparticles, particular tunic cells show phagocytic activity. These phagocytic cells usually have protruding filopodia, often contain some round granules, and occasionally engulf another cell. It is presumed that phagocytic tunic cells migrate throughout the tunic matrix, engulf extraneous substances including bacteria, and also function as scavengers, thereby keeping the tunic free of discarded tunic cells and wound debris.

Cellular defense reactions in the tunic involve two groups of free cells: infiltrating hemocytes that respond to infections or trauma, and tunic cells that always "stand by" in the tunic. In the allogeneic rejection reaction of botryllid ascidians (colonial species belonging to the family Botryllidae, the suborder Stolidobranchiata), many hemocytes infiltrate the tunic from the blood vessels and participate in the necrotic reactions [3, 11]. In *Ciona intestinalis* (a solitary species), when particulate or soluble agents are injected in the tunic, a capsule and/or tissue injury is produced, depending on the dose of the irritant [5, 6, 7]. In this tunic reaction of *C. intestinalis*, the hemocyte infiltration may also occur, induced by the irritant injection, because some of the cells appear around the wound are different from tunic cells [6]. In *Aplidium yamazii*, the phagocytic cells observed here are tunic cells that are always present in the tunic, because they are usually observed in the tunic part of the specimens fixed as whole colony pieces that were not sliced before fixation.

In *C. intestinalis*, De Leo *et al.* [2] studied the fine structure of the tunic and described the "phagocyte" as one type of tunic cell. The characteristics of the "phagocyte" in *C. intestinalis* are very similar to those of phagocytic tunic cells in *A. yamazii*: protruding thin cytoplasmic extensions, phagolysosome-like vesicles, and occasional engulfment of other cells. These phagocytic cells of *C. intestinalis* and *A. yamazii* probably belong to a homologous cell type. In botryllid ascidians, although we have already found amoeboid tunic cells containing bacteria in their vesicles [4], we have not found cells that engulf other cells. The phagocytic tunic cells in *A. yamazii* have some morphological variations in their cell shape and in the number of filopodia (see Fig. 3). These variations are probably caused by different states of phagocytosis, migration, and/or cell differentiation in the same cell type. It is noteworthy that Sawada *et al.* [10] described two types of phagocytes among the hemocytes of the solitary ascidian, *Halocynthia roretzi*: one type (p1-cell) spreads as thin, flat sheets, and the other type (p2-cell) is thicker than the former. P2-cells in *H. roretzi* are similar in morphology to some of phagocytic tunic cells, such as cell "c" in Figure 3, in *A. yamazii*.

In the phagocytic tunic cells, peroxidase activity was demonstrated within the phagolysosomes and/or the vesicles carrying the round granules. Peroxidase is known to inactivate peroxide ions generated in the oxygen-dependent microbicidal pathway. This suggests the following two possibilities: The phagocytic tunic cells may have this microbicidal activity, and the granules in the phagocytic tunic cells are possibly the engulfed materials processed in the phagolysosomes. In contrast to our findings, a cytochemical study using *C. intestinalis* showed that all of the amoebocytes among the hemocytes are peroxidase-negative [8], though the cytochemical methods were different from those of the present study. It is possible that oxygen-dependent microbi cidal activities are not ubiquitous in ascidian tissues or ascidian species.

Based on our observations, we propose a hypothesis on the differentiation of phagocytic tunic cells: 1) The cells with a thin cell body and numerous protruding filopodia are at a young stage. 2) Phagolysosomes are formed after phagocytosis, and their contents subsequently become round granules. The oxygen-dependent microbicidal activity may be carried out during this process. 3) An increase in the number of granules makes the cell body thicker and roundish.

It is thought that almost all tunic cells originate from hemocytes passing through the epidermis. If phagocytic tunic cells originate from hemocytes, the question arises as to whether phagocytic hemocytes migrate into the tunic or hemoblasts differentiate into phagocytic tunic cells within the tunic. For further understanding of the immunological activity and differentiation of tunic cells, a precise description and classification of the tunic cells in *A. yamazii* is required.

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